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# Effects of pH on the structure and function of carboxypeptidase A: Crystallographic studies

(enzyme activity/proteases/protein crystallography/activity of enzymes in solution and crystals)

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**ABSTRACT** **High-resolution crystal structures are described for carboxypeptidase A (EC 3.4.17.1) in crystals grown at pH 8.5, 9.0, and 9.5 and compared with the structure at pH 7.5. The comparison shows that in the pH range of 7.5–9.5 the enzyme structure is practically unchanged, and, most importantly, that the flexible side chain of Tyr-248 remains exclusively in the "up" position, away from the Zn atom, throughout the pH range. There is no evidence for binding of Tyr-248 to Zn at any of these pH values. We conclude that the interaction of Tyr-248 with Zn is not an essential part of the mechanism of carboxypeptidase A and that its occurrence is an artifact of chemical modification of Tyr-248. It is also suggested that Tyr-248 is not uniquely associated with the observed high pK of the enzymatic hydrolysis.**

Carboxypeptidase A (CPA; peptidyl-L-amino acid hydrolase, EC 3.4.17.1) catalyzes the hydrolysis of the carboxyl-terminal residue from peptide or ester substrates by cleavage of the peptide or ester bond. The three-dimensional structure is known to a resolution of 1.54 Å (1). Various mechanisms for the activity of CPA toward substrates have been proposed (2) and recently reviewed (3–7). Of the potential catalytic groups that are in the region of substrate in the x-ray diffraction studies [Glu-270, $L_3{\cdot}Zn{\cdot}OH_2$ (in which L is a protein ligand), $H_2O$, and Tyr-248], we examine here the possible roles of Tyr-248.

Studies of spectral changes of [248-arsanilazotyrosine]-CPA (arsanilazo-CPA) as a function of pH (8, 9) have indicated that the phenolic oxygen of the modified Tyr-248 is bound to the Zn in the unliganded enzyme. On the basis of the results of these studies (8, 9) and related ones (10–18) it has been suggested that the phenolic group of Tyr-248 is directly bound to the Zn atom ("Zn-bound Tyr-248" conformation) in the native unliganded enzyme, and that movement of Tyr-248 *away* from the Zn is an essential step in catalysis of the unmodified enzyme as substrates bind (8–18). In contrast, the crystallographic studies indicate that in the unmodified enzyme the side chain oxygen of Tyr-248 is about 17 Å away from the Zn (1, 2) (the "up" position) and that the phenolic oxygen moves about 12 Å *toward* the active site as substrates bind (2, 19) (the "down" position). Moreover, a study at 1.5-Å resolution shows that at pH 7.5 there is no observable binding of Tyr-248 to the Zn in the unmodified enzyme (1). In a study of the three-dimensional structure of the complex of CPA with the 39-amino acid inhibitor from the potato (PCI), it was shown that cleavage has taken place and that Tyr-248 donates a hydrogen bond to the newly formed carboxylate anion and receives a hydrogen bond from the originally penultimate peptide bond (20). Whether Tyr-248 has other roles such as a proton donor in the cleavage reaction (2–5) or whether formation of a bond between Tyr-248 and Zn is an essential step (8, 9) in the activity of CPA are open to question.

In this paper we report a crystallographic study that extends the earlier study of the native CPA at pH 7.5 (1) to other pH values. We examine the low-resolution (2.8-Å) structure of CPA in crystals in which the pH has been modified in the range of 6–10, and we discuss the refined high-resolution structure of CPA in crystals that have been grown at pH 8.5, 9.0, and 9.5. The study was done on the crystals that are elongated along the *a* axis, or the "*a*-form," for which $a = 51.60$ Å, $b = 60.27$ Å, $c = 47.25$ Å, and $\beta = 97.27°$. These crystals show activity (21) towards 0.02 M carbobenzoxyglycyl-L-phenylalanine of about 1/3 that of the enzyme in solution and of about 100 times that of the crystals that are elongated along the *b* axis, or the "*b*-form," for which $a = 50.9$ Å, $b = 57.9$ Å, $c = 45.0$ Å, and $\beta = 94.7°$. The distinction between these two crystal forms is not always clear in the published literature from other laboratories; a detailed discussion is presented in ref. 22.

The main concern of this report is the conformation of the active site of the enzyme at the various pH values and especially the conformation of Tyr-248 in the native enzyme. We shall address this last point, and we show that Tyr-248 does not bind to Zn in measurable amount between pH 7.5 and 9.5 in the more active form of crystals (the *a*-form).

## MATERIALS AND METHODS

Bovine $CPA_\alpha$ (Cox preparation) was purchased from Sigma and used without further purification. The enzyme in an aqueous suspension was dissolved by dialyzing it against an aqueous solution of 1 M NaCl and 0.03 M Tris·HCl at pH 7.5 in dialysis tubing (Union Carbide) for about 2 days. The solution of enzyme at about 30 mg/ml was filtered (Millipore) and stored at 4°C. This stock solution was used to obtain single crystals at pH 7.5 by dialyzing against 0.2 M NaCl solution buffered at pH 7.5 with 0.03 M Tris·HCl at 20°C in dialysis tubing. We also obtained single crystals at pH 7.5 when 0.03 M sodium cacodylate was substituted for Tris·HCl. Single crystals at pH 8.0, 8.5, 9.0, and 9.5 were grown at 20°C in dialysis wells, by dialyzing against solutions containing 0.03 M Tris·HCl adjusted to the desired pH and containing also, respectively, 0.16, 0.10, 0.07, and 0.04 M NaCl. We also grew single crystals at pH 7.0, 6.5, and 6.0 in dialysis wells by dialyzing at 20°C against 0.03 M sodium cacodylate solutions adjusted to the desired pH and containing respectively, 0.14, 0.11, and 0.05 M NaCl. Our attempts to obtain single crystals at pH 5.5 were unsuccessful. All pH values were adjusted to ±0.1 as measured with a Radiometer (Copenhagen) combined electrode GK2321-C, following cal-

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Abbreviations: CPA, bovine carboxypeptidase A; Mn-CPA, CPA in which Mn is substituted for Zn.

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ibration with standard buffers (Fisher). Crystals appeared after 3 days to 5 weeks and were allowed to grow to dimensions of about $1 \times 0.5 \times 0.5$ mm. Another series of pH crystals were obtained by gradually changing the pH of a buffered solution in which crystals were present from 7.5 to the final pH over a period of 3–6 days. These "soaked" crystals of high quality were obtained at pH values of 6.0, 6.5, 7.0, 8.0, 8.5, 9.0, 9.5, and 10.0. We verified the pH to be within ±0.2 of the desired value before mounting each crystal for x-ray diffraction.

X-ray diffraction data were collected on a Nicolet $P2_1$ automatic diffractometer using Cu $K_\alpha$ radiation ($\lambda = 1.54178$ Å) with a graphite monochromator. The crystals were mounted in a glass capillary with more than the usual amount of mother liquor in order to maintain the buffer. Thin strips of pH-sensitive paper (Micro Essential) indicated that no significant changes in pH occurred after mounting. Unit cell dimensions (22) for crystals above pH 7.5 were similar (within ±0.3%) to those given above for the crystals at pH 7.5. At pH 9.5 the unit cell dimensions are $a = 51.93(3)$ Å, $b = 60.35(3)$ Å, $c = 47.05(3)$ Å, and $\beta = 97.17(4)°$. While crystals above pH 7.5 are isomorphous with those at pH 7.5, we found that weak superlattice reflections occurred at pH 7.0, 6.5, and 6.0. No attempt was made to solve the detailed structure of this larger unit cell, in which slight changes occur in the molecular packing. Also, at these low pH values (6.0–7.0) slight changes in the cell dimensions, particularly in the *a* axis (elongation by about 0.5–2%) and the *c* axis (shortening by about 1–2%) were noted; at pH 6, the cell parameters are $a = 52.43(2)$ Å, $b = 60.48(3)$ Å, $c = 46.36(3)$ Å, and $\beta = 97.32(3)°$, if one ignores the superlattice. Similar altered crystal forms have been observed upon diffusion of some inhibitors and substrate analogs into CPA crystals and also in crystals of CPA that were grown or soaked in a number of aqueous/organic mixture solutions (unpublished results). The diffraction data were collected with a Wyckoff step-scan (23), at speeds of 2.0 to 0.6 deg/min. Collection of data was terminated when the decay of intensities of four check reflections exceeded 20%. A linear correction for radiation damage was made, based on intensities from these check reflections, and the usual Lorentz, polarization, and absorption (24) corrections were also made.

Intensity data to 2.8-Å resolution (about 7000 independent reflections) were collected on crystals either grown or soaked at pH values of 6.0, 6.5, 7.0, 8.0, 8.5, 9.0, and 9.5. We have not observed any significant differences between crystals that were grown and crystals that were soaked in any particular pH. Difference electron density maps between each of these and the data at pH 7.5 showed no major features. For the difference map calculations, the superlattice reflections from crystals at low pH were omitted from the data. We conclude that there are no major structural changes throughout this pH range. Inasmuch as the crystals at low pH diffract poorly beyond 2.5-Å resolution, we continued to higher resolution only with the crystals grown at pH values of 8.5, 9.0, and 9.5, from which we collected data to limiting resolutions of 1.50 Å (26,786 unique reflections), 1.64 Å (17,075 unique reflections), and 1.82 Å (14,682 unique reflections), respectively.

After a detailed examination of difference electron density maps, which indicated no major changes relative to the structure at pH 7.5, the structures were refined at each pH by a restrained least-squares method (25, 26). Computation of the structure factor derivatives by using a difference map algorithm (27) reduced the computational time by about an order of magnitude. The initial model (1) contained 2437 nonhydrogen atoms of the protein, 1 Zn atom, and 191 water molecules. We deleted solvent molecules in the active site region, and added them after inspection of difference electron density maps. Residue conformations were examined during the refinement, using maps computed with Fourier coefficients ($2F_o - F_c$) and calculated phases. No adjustments to the protein side chains appeared to be necessary. All refinements converged smoothly to final crystallographic *R* factors [$R = \Sigma||F_o| - |F_c||/\Sigma|F_o|$] of 0.189, 0.169, and 0.175 for the pH 8.5, pH 9.0, and pH 9.5 CPAs, respectively. The details of the refinements and deviations of the refined structures from ideal geometry are summarized elsewhere (22).

## RESULTS

The final refined structures have been compared to the refined native CPA structure (1) and exhibited overall rms deviations of 0.14 Å, 0.21 Å, and 0.23 Å, for the pH 8.5, pH 9.0, and pH 9.5 CPAs, respectively. The individual rms deviations for the protein residues in or around the active site and for some of the residues that deviate most from the rest of the structure, are listed elsewhere (22). Very little movement of the polypeptide backbone was seen, and no side chain moved by more than 0.9 Å relative to its position at pH 7.5. A number of non-active-site side chains deviate with increasing pH; most of them are charged or polar residues (as expected in an increasingly ionizing medium). Nonetheless, the majority of these deviating residues are on the surface of the molecule, 15 Å or more away from the active site, and do not seem to be correlated with the enzyme pK or with any indirect effect related to the enzymatic activity. In particular, the two side chains that exhibit the highest deviations at high pH values, Ser-159 and Thr-293, are located more than 25 Å away from the binding site, on the surface of the molecule and in one of the $\alpha$-helices in the other side of the protein, respectively. The only two "noncatalytic" side chains that show significant deviation at higher pH values and could be related indirectly to pH–activity relationships are Lys-239 and Ile-255. The Lys-239 side chain is hydrogen bonded to a main chain segment that is 2 residues away from Tyr-248, and Ile-255 is near the hydrophobic "pocket." Nevertheless, it seems unlikely that such remote movements would affect the enzyme activity at high pH. Threonine residues are often found disordered or conformationally populating two rotamers (28–30) in protein molecules due to a low energy barrier for rotation of $\chi^1$. Thus, their reorientation is not unexpected (22).

In the active site, Tyr-248 (in the up position, away from the Zn atom) did not shift at all at the different pH values. The movement of Zn (about 0.05 Å) is insignificant. The temperature factors of both Tyr-248 (average *B* values of 16–20) and Zn (*B* values of 3–5) have also remained relatively unchanged. On the other hand, slight changes occurred in the positions of two ligands to Zn, His-69 and His-196, at the higher pH values (ref. 22 and Fig. 1). Also, the positions of the Zn-bound water (567) and the next nearest water (571) changed slightly. These apparent changes in the relative positions result in slight shifts in the geometry of the active site (Fig. 1 and Table 1). In view of our estimated standard deviation of at least ±0.2 Å in atomic positions, we are reluctant to assign any chemical significance to these small structural changes. In particular, it would be dangerous, however tempting, to consider these changes as support for ionization of the $L_3{\cdot}Zn{\cdot}OH_2$ complex. Moreover, these slight movements do not change the coordination about Zn, which remains liganded to the protein through His-69, His-196, and both oxygen atoms of Glu-72 at pH values of 7.5, 8.5, 9.0, and 9.5. Water 571 appears to move closer to Zn at the higher pH values, but the short distance of 1.6 Å at pH 9.5 is abnormal and may reflect larger errors due to the smaller number of reflections of the high pH crystals. Also, at the higher pH values, the temperature (disorder) factor for water 571 decreases from $B \approx 30$ at pH 7.5 to $B \approx 4$ at pH 9.5, suggesting higher occupancy, while the temperature factor

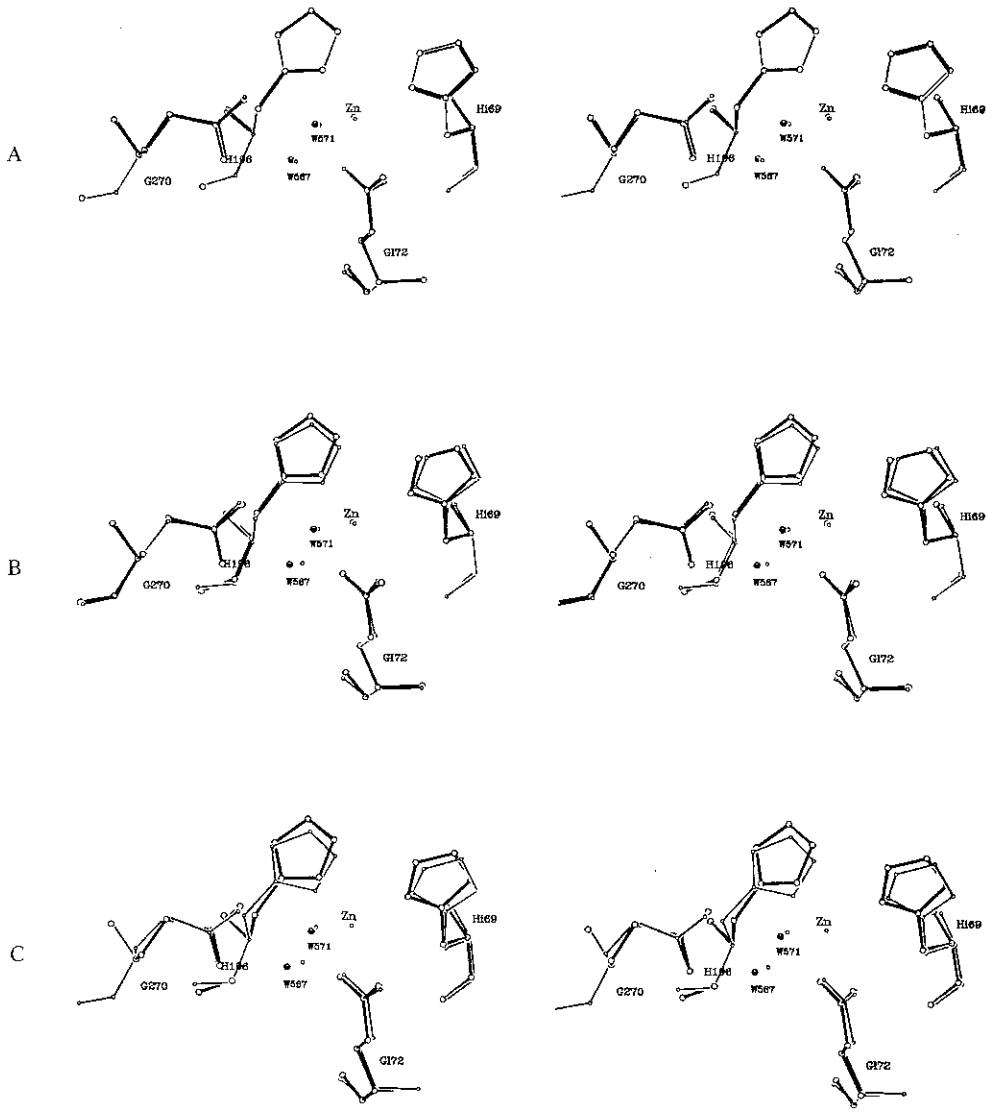


FIG. 1. Stereoviews of the Zn environment of pH 8.5 CPA (*A*), pH 9.0 CPA (*B*), and pH 9.5 CPA (*C*), superimposed on the corresponding site of the native CPA (pH 7.5). The pH structures are presented in thin lines and the native structure is presented in thicker lines. Water 567 and water 571 are represented as dark circles in the native and as open circles in the pH structures.

for water 567 remains relatively unchanged. Finally, a slight rotation of some 20° may occur around the $C^{\gamma}$—$C^{\delta}$ bond of Glu-270 at the high pH values.

## DISCUSSION

**Conformation of Tyr-248.** The main point of this study is that at any of these pH values (7.5–9.5) we find no evidence for a conformation of Tyr-248 in the down position, which is observed upon binding of analogs and inhibitors (2, 19, 20, 31), or for a conformation in which the phenolic group is directly bound to the Zn atom ("Zn-bound Tyr-248" conformation), as suggested from spectroscopic data of CPA derivatives in which the Tyr-248 side chain had been chemically modified (7–9). A more direct approach, in which we started with Tyr-248 in both the up and Zn-bound positions, resulted in least-squares refinement of occupancies of <5% in the Zn-bound position and ≈100% in the up position. The absence of any continuous density for the tyrosine ring in the Zn-bound position leads us to suggest that the most probable value of this orientation is zero, although our limit of error is about ±5%.

We cannot, of course, be absolutely sure that some constraints of molecular packing inhibit Tyr-248 from binding to $Zn^{2+}$. However, our procedure of growing the crystals at each of several pH values (7.5, 8.5, 9.0, and 9.5) is expected to decrease the influence of packing as compared with a procedure of changing the pH of a crystal that is already grown. Nor can we be certain that the molecular structure of the enzyme is unaffected by crystallization (22). However, in both the structures of the native CPA at pH 7.5 (1) and those of the higher-pH CPAs, no significant intermolecular interactions involving Tyr-248 are present that could possibly restrict the flexibility of this side chain. Tyr-248 is in van der Waals contact with side-chain atoms of Thr-14 and His-120 in a neighboring molecule (1). However, no intermolecular hydrogen bonds to the phenolic hydroxyl of Tyr-248 are observed; this group is hydrogen bonded to a water molecule (19), and this bond remains intact at high pH values. Additionally, the temperature factors of the atoms in the side chain of Tyr-248 at any pH do not show the smaller values that are typical of hydrogen-bonded or constrained side chains. Moreover, the phenolic group of Tyr-248 is demonstrated to move down toward the Zn atom, although not to coordinate with it, upon binding of substrate analogs and other ligands to the active site (2, 19, 20). These conformational changes occur during the diffusion of ligands into the *same crystals* used here. The data presented above strongly indicate that Tyr-248 is relatively constraint free in the crys-

Table 1. Geometry of the active site in native CPA and CPAs at various pH values

| Atoms | Native CPA | pH 8.5 CPA | pH 9.0 CPA | pH 9.5 CPA |
|---|---|---|---|---|
| | Distance, Å | | | |
| His-69 $N^{\delta 1}$–Zn | 2.1 | 2.1 | 2.3 | 2.3 |
| Glu-72 $O^{\varepsilon 2}$–Zn | 2.3 | 2.3 | 2.4 | 2.2 |
| Glu-72 $O^{\varepsilon 1}$–Zn | 2.2 | 2.3 | 2.0 | 2.3 |
| His-196 $N^{\delta 1}$–Zn | 2.1 | 2.3 | 1.9 | 1.9 |
| Water 571–Zn | 2.1 | 1.8 | 1.7 | 1.6 |
| Water 567–Zn | 3.2 | 3.3 | 3.4 | 3.4 |
| Water 571–water 567 | 1.5 | 1.7 | 1.9 | 2.1 |
| Water 571–Glu-72 $O^{\varepsilon 2}$ | 3.6 | 3.3 | 3.4 | 3.2 |
| Water 571–Glu-72 $O^{\varepsilon 1}$ | 3.2 | 3.1 | 2.9 | 3.0 |
| Water 571–His-196 $N^{\delta 1}$ | 3.1 | 3.0 | 2.6 | 2.3 |
| Water 571–Glu-270 $O^{\varepsilon 2}$ | 3.2 | 3.2 | 3.3 | 3.4 |
| Water 571–Glu-270 $O^{\varepsilon 1}$ | 2.5 | 2.7 | 2.8 | 2.9 |
| Water 567–Glu-270 $O^{\varepsilon 2}$ | 2.3 | 2.5 | 3.0 | 3.2 |
| Water 567–Glu-270 $O^{\varepsilon 1}$ | 2.5 | 2.7 | 2.9 | 2.7 |
| | Angle, deg | | | |
| His-69 $N^{\delta 1}$–Zn–Glu-72 $O^{\varepsilon 2}$ | 121 | 121 | 123 | 112 |
| His-69 $N^{\delta 1}$–Zn–Glu-270 $O^{\varepsilon 1}$ | 91 | 93 | 99 | 94 |
| His-69 $N^{\delta 1}$–Zn–His-196 $N^{\delta 1}$ | 99 | 102 | 97 | 104 |
| His-69 $N^{\delta 1}$–Zn–water 571 | 121 | 126 | 128 | 135 |
| Glu-270 $O^{\varepsilon 2}$–Zn–His-196 $N^{\delta 1}$ | 101 | 92 | 94 | 92 |
| Glu-270 $O^{\varepsilon 2}$–Zn–water 571 | 111 | 108 | 108 | 111 |
| Glu-270 $O^{\varepsilon 1}$–Zn–His-196 $N^{\delta 1}$ | 156 | 148 | 150 | 149 |
| Glu-270 $O^{\varepsilon 1}$–Zn–water 571 | 98 | 99 | 99 | 102 |
| His-196 $N^{\delta 1}$–Zn–water 571 | 96 | 95 | 91 | 83 |

tal form used in our studies (22). We therefore conclude that since no Zn-bound Tyr-248 is observed at any pH between 7.5 and 9.5, this conformation does not exist, or it is insignificantly populated at this pH range, implying that coordination of the phenolic hydroxyl is not an important feature of the resting enzyme and is definitely not a required conformation for activity of CPA, as has often been suggested (8–18).

As for the location of the chemically modified side chain of Tyr-248 in arsanilazo-CPA and [248-*o*-nitrotyrosine]CPA (nitro-CPA), it is quite possible that the predominant conformation of this residue is that referred to above as the Zn-bound Tyr-248 conformation (8, 9, 18) or other altered conformations in which the phenolic group is brought to close proximity with a positive charge (13, 14), both in the crystal and in solution. However, considering the crystallographic results presented above, we conclude that direct bonding of Tyr-248 to Zn (or any other altered conformation) is favored by the *chemical modification* itself and that this conformation is neither a common nor a required feature for the active enzyme. This conclusion is additionally supported by the finding that nitration of Tyr-198 has not been observed in the native enzyme, although the enzyme "becomes susceptible to modification only as a consequence of a conformational change that accompanies azo coupling of Tyr-248" (32). The arsanilazo-Tyr-248 derivative retains about 1/3 of the activity of CPA at low substrate concentrations, where substrate anomalies are reduced (33). The question of the similarity between the conformations of native and chemically altered CPA in solution and in the crystal is discussed elsewhere (22).

**pK Assignments.** CPA is known to be reasonably active only in the pH range of approximately 6–9, with an optimum around pH 7.5 (34). Several kinetic studies (35–43) relate to the details of pH–activity relationships in the hydrolysis catalyzed by CPA and contain attempts to assign the usually observed high (≈9) and low (≈6–6.5) pKs to specific catalytic groups (44, 45). Although these kind of pK assignments are usually complicated and may be misleading (46), we present here a limited discussion. For "normal" peptides, those that minimize kinetic anomalies, the pH dependence of $k_{cat}/K_m$ indicates pK values of roughly 6.2 and 9.0, whereas the pH dependence of $k_{cat}$ exhibits a low pK in the range of 6.0–6.3 but no second pK below pH 10.5 (35–37). Esters appear to be divided into two groups—those with a pH dependence similar to amides [e.g., the *trans*-cinnamoyl esters (38, 39)] and those with a different pH dependence, which is often characterized by a second pK around 8–9.4 in the $k_{cat}$ vs. pH profiles (40–43). The low pK could be assigned to the ionization of either Glu-270 (47, 48) or the Zn-bound water molecule (6, 49), and the high pK could be assigned to the ionization of Tyr-248, Tyr-198, or the Zn-bound water (3, 6, 48).

Our present results indicate that Tyr-248 and Tyr-198 do not appear to move, and the arrangement of water molecules around them does not seem to change, in the pH range of 7.5–9.5. Of course, it is possible, although unlikely, that the ionization of Tyr-248 is not accompanied by changes in the structure of the protein or water. In the free enzyme the phenolic oxygen of Tyr-248 remains 17 Å from the Zn atom. Also, the high limb of the pH profiles, as examined for an ester substrate, remains unchanged upon nitration and diazotization of Tyr-248 (and probably also Tyr-198) (39). One could expect these chemical modifications to affect significantly the pK of a tyrosine residue. Moreover, acetylation of Tyr-248 has been shown to have little effect on the enzymatic hydrolysis of *O*-(*trans*-cinnamoyl)-L-β-phenyllactate (50), suggesting that Tyr-248 may not play a significant role in the hydrolysis of this substrate. In contrast, it has been shown that nitration of Tyr-248 considerably affects the apparent enzymatic high pK, which is decreased to about 6.7, when a peptide substrate is examined (51), although the nitro-CPA retains full activity toward such a substrate in the lower pH range.

In summary, these results indicate that Tyr-248 (and probably also Tyr-198) is not the ionizing group primarily responsible for the high pK of ≈9 of the native uncomplexed enzyme, at least for esterase activity.

There are some tentative indications that the Zn-bound water has a high pK. Previous crystallographic studies have demonstrated that the Zn-bound water has to be displaced for substrate analogs to bind to the Zn (2, 19, 20, 31). This binding would become more difficult if Zn-bound hydroxide ion had to be displaced. The pK values for Zn-bound water observed in small model compounds (pK ≈ 9–10) (52, 53) and the comparable temperature variation of the high pK of CPA (from the $k_{cat}/K_m$ vs. pH profiles) and water bound to a metal in model compounds (37, 52) are both consistent with this pK assignment. Additional support for the assignment of the high pK to the metal-bound water comes from CPA in which Mn was substituted for Zn (Mn-CPA). Mn-CPA has been shown to be catalytically active (54), and crystallographic studies showed that its structure is practically identical to the native Zn-CPA (unpublished results); in Mn-CPA one water is 2.2 Å from Mn, while a second water molecule is 3.7 Å from Mn (unpublished results). NMR studies in solution on Mn-CPA showed that the metal is coordinated to at least one water molecule (and possibly two) in the uncomplexed enzyme at the active pH range, and that the metal-bound water is displaced by a binding inhibitor (55). NMR dispersion experiments with Mn-CPA indicated that there is no change in proton ionization from the hydration shell of Mn in the pH range of 8–9 (56), while kinetic measurements on Mn-CPA exhibited a high apparent pK of 9.3, which has been interpreted as the ionization of the $Mn{\cdot}H_2O$ complex to $Mn{\cdot}OH^-$ (50). In Co-CPA there is a spectral inflection point at pH 8.8 (57). The lack of a pK on the high side of the $k_{cat}$ vs. pH profile [related to the pK of the enzyme–substrate complex (44–46)] also favors the Zn-bound water assignment

since this ionizing group is not present in the enzyme–substrate complex. Thus either the Zn-bound water has been displaced by the substrate or substrate does not bind to the deprotonated (Zn-OH) enzyme (48).

On this ground the relative metal insensitivity of the apparent high pK of CPA (36) may be somewhat surprising. However, the pKs of metal-bound water observed for complexes of Zn, Co, and Mn in model compounds are in the same range of 9–10 (53). The deacylation of what may be the covalent acyl-enzyme intermediate for an ester substrate at subzero temperature indicates the involvement of a group with a pK at −35°C of about 7.5 for the Zn enzyme and about 6.2 for the Co enzyme (49). A self-consistent interpretation is that when Glu-270 is unmodified it maintains a high pK for the $Zn{\cdot}OH_2$ moiety in spite of the proximity of Arg-127, and that when Glu-270 is covalently bound the pK of $Zn{\cdot}OH_2$ is lowered by the neighboring arginines, especially by Arg-127, and by a slight increase in the hydrophobic character of the environment. While this analysis does suggest a basis for a lowering of the pK of $Zn{\cdot}OH_2$ when an anhydride intermediate is found, we remain uncertain as to whether some ester and peptide substrates proceed through this acyl-enzyme pathway or through the general base pathway in which Glu-270 promotes the attack of a water molecule at the carbonyl carbon of the scissile bond of the substrate. Further implications of the tentative pK assignments presented above regarding possible mechanisms for CPA catalysis are discussed elsewhere (22).

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